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Mutational landscape of *DDR2* gene in lung squamous cell carcinoma using next-generation sequencing

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Micro-abstract:

DDR2 alterations were identified as a promising therapeutic target in lung SCC. Using a large cohort, *DDR2* genetic landscape was assessed by next-generation-sequencing. *DDR2* mutations are present in 4% of SCC, are not exclusive with driver genes alterations, and have no prognosis impact. This study emphasizes the need for a better knowledge of *DDR2* biology before developing dedicated targeted therapies.

Abstract:

Background: Lung cancer represents the leading cause of cancer-related death worldwide. Despite great advances in lung cancer management with the recent emergence of molecular targeted therapies for non-squamous non-small cell lung cancer, no dramatic improvements have been achieved in lung squamous cell carcinoma (SCC). Mutations in *DDR2* gene were recently identified as promising molecular targets in this histology. The aim of this study is to describe the *DDR2* mutational landscape of lung SCC and investigate the associated clinical factors.

Methods: Next generation sequencing of the *DDR2* gene was performed on 271 samples of lung SCC. Patients followed in our institution from January 2011 to August 2014 were retrospectively selected for data collection. Other driver gene alterations (*EGFR*, *KRAS*, *BRAF*, *HER2* and *PI3KCA*) were analyzed using pyrosequencing.

Results: A total of 11 patients harboring a *DDR2* mutation was detected among the 271 sequenced lung SCC samples (4%). We describe 10 unreported mutations, comprising a novel *DDR2* exon 7 splice mutant. *DDR2* mutations were not mutually exclusive with other driver gene alterations. One hundred thirty-six patients were included for clinical comparison and logistic regression analysis. No difference was detected between *DDR2*-mutant and *DDR2* wild-type lung SCC regarding clinical characteristics or survival.

Conclusion: *DDR2* mutations were observed in 4% of lung SCC cases of European descent. *DDR2*-mutated tumors can exhibit other driver gene alterations. No clinical characteristics were significantly associated with *DDR2* mutation.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, accounting for more than 1.5 million deaths in 2012.¹ Non-small cell lung cancers (NSCLCs) represent approximately 85% of lung neoplasms; among these, squamous cell carcinomas (SCC) account for approximately 30% of cases. The landscape of thoracic oncology has greatly changed during the last decade with the discovery of tumor driver mutations predicting response to targeted therapies.² Recent clinical trials demonstrated an unprecedented improvement in progression-free survival (PFS) and quality of life, especially in molecularly selected lung adenocarcinoma.³⁻⁵ Lung SCC lacks available molecular targets, and tyrosine kinase inhibitors are mostly ineffective in unselected populations⁶ despite many efforts made to understand its genomics.⁷ Among all of the somatic alterations explored to date in lung squamous histology, Discoidin Domain Receptor 2 (*DDR2*) gene mutations are reported in approximately 3% of cases.^{8,9} *DDR2* is a tyrosine kinase receptor that belongs to the same family as Epidermal Growth Factor Receptor (EGFR).¹⁰ Numerous *in vitro* studies on various tumoral cell lines demonstrated the important role of *DDR2* in the regulation of cellular proliferation, migration, metastasis, and secretion of matrix metalloproteinase.¹¹ In 2011, Hammerman *et al* showed that a subset of *DDR2* mutants are oncogenic in SCC cell lines *in vitro*, delivering a strong rationale for targeting *DDR2* mutations.⁸ In addition, two patients with *DDR2*-mutated lung SCC were reported in the literature to experience a dramatic response to dasatinib, a potent Src inhibitor.^{8,12} Altogether, these data establish *DDR2* mutation as a promising molecular target of tyrosine kinase inhibitors in lung SCC.

Few studies are available concerning the clinical characteristics of patients harboring *DDR2* mutations. The first aim of this study was to describe *DDR2* gene mutations in a large monocentric cohort of SCC. Further objectives of the study were to compare the clinical characteristics of *DDR2*-mutant to *DDR2* wild-type (WT) lung SCC and to investigate factors associated with *DDR2* mutations.

Patients and methods

Tissues and patients

Samples were obtained from patients who were diagnosed with SCC by tissue biopsy or primary surgical resection from January 2011 to January 2015 in five hospital centers in Brittany (north-west of France). A total of 271 lung SCC samples were retrospectively enrolled for *DDR2* gene sequencing on a centralized platform. For demographics, clinical and survival comparisons, we chose to only select patients diagnosed and followed from January 2011 to August 2014 in Rennes University Hospital to avoid site-dependent care bias and limit retrospective data missing in patient's record. The choice of this recruiting period allows a minimal follow-up of at least 12 months for all patients. Subsequently, a total of 136 patients with lung SCC composed the investigation cohort (appendix 1). Central localization of the tumor was defined as proximal to the third bronchial division and advanced disease as unresectable stage IIIB or stage IV tumors according to the 7th IASLC TNM classification for lung cancer.¹³ Concerning the smoking exposure, patients were classified as active smoker, former smoker (no exposition during the last 12 months at diagnosis), never-smoker or unknown. The study was approved by the Rennes University Hospital Ethics Committee (n° 15.87).

DDR2 gene sequencing, sequence processing, variant calling and analysis of *DDR2* mutations

All tumor tissues were subjected to review by a pathologist to estimate the tumor cell content and 10 μ M-thick formalin-fixed paraffin-embedded tissue sections were sampled. A tumor cell cut-off of at least 10% was used to proceed further with the sample. Normal tissue was also sampled when matched non-tumoral sequencing was indicated. Genomic DNA was extracted from routine FFPE samples using the MagDEA DNA 200 kit (Precision System Science®, Japan). Genomic DNAs from all samples were quantified with the Quan-iT PicoGreen dsDNA assay kit (Invitrogen). A panel of 36 specific primers targeting the *DDR2* gene was created using AmplifX (see appendix 4 for sequence). All primers were validated by single-plex PCR and assessment of PCR products for expected size was performed on LabChip Gx (Perkin Elmer®). The experiments were performed according to the Multiplex Amplicon Tagging Protocol from the manufacturer (Fluidigm®) in three major steps: (i) a

multiplex specific target pre-amplification, (ii) a target enrichment PCR on the Access Array IFC and (iii) a barcode PCR. All PCRs were performed using FastStart High Fidelity PCR System, dNTPack (Roche®). The libraries' quality and concentration were evaluated on a LabChip GX (Perkin Elmer®). Next, libraries were pooled together to create one PCR product library. The resulting sequencing-ready amplicon libraries were sequenced on a MiSeq sequencer (Illumina) using MiSeq Reagent Kit v2 (300 cycles). Each patient was sequenced twice with independent amplicon library preparation. The bioinformatics pipeline to detect low frequency variants developed for molecular testing in a diagnosis purpose was used to detect alterations in the *DDR2* gene. Briefly, reads were aligned to the human reference genome GRCh37 (hg19) using BWA software (bwa-sw 0.5.9), recalibrated and realigned using GATK (GATK-2.1-11). NGS amplicon reads were split into non-overlapping bam files. These bam files were used to detect single-nucleotide variants and insertions/deletions based on SAMtools mpileup files (samtools-0.1.18). Custom scripts were used to identify sequence variants in the pileup files. For each patient at each variant position, variant allele fractions (VAFs) were estimated separately on forward and reverse reads, by amplicon and by index. When calling the variants, the minimum read depth was set to 150 in forward and reverse reads and in both indexes (i.e. a total read depth ≥ 600) and the variant allele must be present in $> 10\%$ of both reads. Variants were annotated with ANNOVAR and selected when present inside exons or at canonical splice sites. The genome version used was grch37/hg19 (February 2009) and the Refseq for *DDR2* gene was NM_006182.3. Integrative Genomics Viewer (IGV) was used to visualize the read alignment and to confirm the variant calls. If the *DDR2* variant allele was $>50\%$, systematic *DDR2* gene sequencing of normal tissue was performed to confirm the somatic status of the nucleotide variation. Potential protein-altering mutations were analyzed using CRAVAT 4.3 software.^{14,15} Pre-mRNA splicing potential defects were studied using the Human Splicing Finder 3.0 website.¹⁶

Detection of *EGFR*, *PI3KCA*, *KRAS*, *BRAF* and *HER2* mutations

Genomic DNA was isolated from FFPE tissue specimen using the MagDEA DNA 200 kit (Precision System Science, Japan) according to the manufacturer's instruction. DNA samples were quantified using a Nanodrop spectrophotometer and normalized to 10 ng/ μ l. Mutations of *EGFR* (exons 18–21),

HER2 (exon 20), BRAF (exon 15), KRAS (exon 2), and PIK3CA (exons 9 and 20) were detected on a routine basis on the biomolecular platform of Rennes University Hospital, using respective Refseq : EGFR: NM_005228.3; HER2: NM_004448.3; BRAF: NM_004333.4; KRAS : NM_033360.3 and PIK3CA: NM_006218.2. The methodology was based on pyrosequencing assays, as previously described.¹⁷ ALK and FGFR genes fusions were not include in the analysis as mainly not available in our cohort (not routinely assessed at the time of inclusion).

Statistical analysis

Demographic and descriptive data are given as the median with the range. Categorical variables were compared with the Fischer exact test or Pearson chi-squared test, and quantitative variables were compared with the Mann-Whitney U test when appropriate. Overall survival was calculated in months from the date of diagnosis to the date of death (failure) or the date the patient was last known to be alive (censored). Two-tailed p-values were reported, with $p < 0.05$ considered as statistically significant. The Kaplan-Meier method with log-rank test were used to perform survival analysis. Finally, a binomial logistic regression model was built with the *DDR2* status (mutant or wild-type) as the dependent variable. GraphPad Prism® for Windows software (version 5.03; Graph Pad Software Inc., Los Angeles) was used for the statistical and survival analyses.

Results

Frequency, distribution and characteristics of lung SCC DDR2 mutation

Next generation sequencing was performed on 271 samples of lung SCC. Percentage of DDR2 gene coding sequence coverage was 100%, with a minimum of 600X depth for each sample (see appendix 5). *DDR2* mutations were found in 4% (11/271) of the cases. Table 1 summarizes *DDR2* mutations characteristics. The majority of *DDR2* point mutations observed were nucleotide transversions (73%). Prediction of the functional impact on the DDR2 protein reveals that four mutations had a neutral effect whereas seven showed a deleterious effect. *DDR2* mutations predominantly map onto the discoidin homology domains and the kinase domain, each comprising 36% of the mutations (Figure 1A). Only one *DDR2* mutation (c.1857G>C, p.R619S) described in this study was previously reported in lung SCC.¹⁸ A new *DDR2* splice mutation was identified: c.566-1G>C. This substitution occurring in the late intron upstream of exon 7 of the *DDR2* gene alter the wild-type acceptor site. A new splicing acceptor site is predicted at position c.564 resulting in a reading frame shift, as schematized in Figure 1B. Of note, single nucleotide polymorphism (SNP) of the *DDR2* gene was found in seventeen patients in our cohort (according to public database or paired sequenced non-tumoral tissue) (see appendix 2).

Mutational status of *EGFR*, *PI3KCA*, *KRAS*, *HER2* and *BRAF* genes

In our panel, the mutational status for other known driver genes was routinely assessed for 265 patients. Seventeen patients had a mutation in one of these genes, including five patients with an Exon 2 c.34G>T *KRAS* mutation, one patient with an exon 20 c.2338C>A *HER2* mutation, eleven patients with a mutation *PI3KCA* (ten with exon 10 c.1624G>A and one with exon 21 c.3140A>G). These results are summarized in Table 2. One tumor exhibits a concomitant deleterious p.G575R *DDR2* mutation and a p.G12D *KRAS* mutation.

Investigation cohort characteristics

Demographic and descriptive statistics of the *DDR2*-mutant and the *DDR2*-WT groups of the investigation cohort are listed in Table 3 and clustered genomic profile of the investigation cohort is presented in figure 2. The two groups were composed of male heavy smokers (81% and 93% tobacco exposure in the *DDR2*-mutant and the *DDR2*-WT groups, respectively) with a high level of comorbidities, mainly cardiovascular disease, in approximately two-thirds of the cases in the entire cohort. No differences in age, gender, smoking history, pack-year, comorbidities, Eastern Cooperative Oncology Group (ECOG) performance status, disease stage or follow-up were observed between the two groups.

Factors associated with *DDR2* mutation and prognosis impact of *DDR2* mutations

Performing logistic regression, we investigated factors associated with *DDR2* gene mutational status. In univariate analysis, no variables were significantly associated with *DDR2* mutant lung SCC (see appendix 3). Global cohort survival analysis using the Kaplan-Meier estimator showed no difference between *DDR2*-mutant lung SCC and *DDR2*-WT lung SCC ($p=0.71$; HR : 0.52 CI 95% [0.25-1.08]) (Figure 3A). A subgroup comparison of advanced *DDR2*-mutant lung SCC with advanced *DDR2*-WT lung SCC revealed no difference concerning overall survival ($p=0.77$; HR : 1.66 CI 95% [0.75-3.71]) (Figure 3B) or progression-free survival ($p = 0.65$; HR: 0.76 CI 95% [0.28 to 2.08]) (data not shown).

Discussion

In this study, we provide one of the largest description of *DDR2* genetic landscape in lung SCC to date. We identified eleven *DDR2* mutations (4%) and report for the first time, a splicing mutant c.566-1G>C of the *DDR2* gene in lung cancer. The following rates of mutation were previously observed: 2% (2/100), 3.2% (9/277), 4.6% (4/86), 1.1% (2/178), and 1.3% (3/178) in the lung SCC cohorts of Lee *et al*¹⁹, Hammerman *et al*⁸, Miao *et al*²⁰ and cBioportal database (TCGA⁷ and TCGA provisional) respectively. In contrast, Kenmotsu *et al*²¹ (sequencing limited to p.S768R mutation), and Hashima *et al*²² (sequencing of 8 exons of *DDR2* gene) failed to identify any *DDR2* mutation in their Japanese cohort comprising more than one hundred cases. Of note, sequencing coverage of *DDR2* gene is very different between these studies and may contribute to such prevalence inconsistency. Some authors have suggested that *DDR2* mutations may be related to tobacco exposure.²³ To support this notion, the largest percentage of *DDR2* mutations described in this report are transversions, known to be smoking-related.²⁴ Epidemiologic studies showing that East Asian populations are less susceptible to smoking-related lung cancer²⁵ might also partially explain these geographic variations.

We identified a new *DDR2* truncating mutation p.E85X that is predicted to have a major impact on the receptor function. Nevertheless, to date, some discrepancies exist in the literature concerning the oncogenic potential induced by *DDR2* aberrant signaling. Functional validations assays are needed to assess the oncogenic properties of this variant. Moreover, *DDR2* mutations appear not to be exclusive from other driver gene alterations. This is supported by the observation of co-occurrence of such mutations with a *KRAS* p.G12D mutation in our cohort. However, known-driver mutations frequencies appear lower in our cohort than expected, comparing to TCGA⁷. This could be explained by our methodology based on hotspot exon sequencing which contrary to TCGA, do not cover all gene coding-sequence. In their founding publication, Hammerman *et al* demonstrated that ectopic expression of a subset of *DDR2* mutants (p.L63V and p.I638F) in non tumoral cells is able to promote proliferation *in vitro* in a *DDR2*-dependent manner⁸ These results were strengthened by *in vivo* experiments with a recent publication revealing that inducible *DDR2* p.L63V mutation combined with

conditional loss of *TP53* is oncogenic in a murine model.²⁶ However, a recent publication showed the presence of *DDR2* p.L63V and p.G505S alterations not only in tumor specimens but also in matched normal tissues, suggesting that these variants are inherited.²⁷ Some of these variants, also identified in our cohort, have already been reported in the NHLBI Exome Sequencing Project²⁸ but misclassified as mutations in the literature. Further research should now investigate to what extent these constitutional *DDR2* variants could be associated with lung SCC initiation; similar to the ongoing research on *EGFR* germline mutations.²⁹ A better deciphering of *DDR2* somatic mutations by deep-sequencing is now critical to better select patients in clinical trials, as new potent and selective *DDR2* inhibitors are currently in development.³⁰

Some limitations to our study should be noted. First, the sample size of our study was limited. As *DDR2* mutations are rare in lung SCC, it is possible that the power of our study was insufficient to detect potential associated factors. Second, a significant proportion of the 271 sequenced patients had to be withdrawn from the data collection to avoid missing information. Consequently, potential selection bias could have influenced our findings. Third, this study was monocentric, and reproducibility of the molecular results could not be assessed on multiple genomic platforms. Fourth, we cannot definitively confirm the non-germline status of one of the new p.P492S *DDR2* variant described in this cohort, as samples with healthy tissue were not available for this patient.

Conclusion

In conclusion, *DDR2* mutations were detected in 4% of cases in our lung SCC cohort. Our study identified ten new *DDR2* mutations that have not been described to date, including a unique *DDR2* splice mutant and shows that *DDR2*-mutated tumors can exhibit other driver gene alterations. No clinical characteristics or survival difference was detected between *DDR2*-mutant and *DDR2*-WT lung SCC, and no clinical factors were significantly associated with *DDR2* mutation. A better understanding of *DDR2* biology and its mutants will be critical for developing future dedicated therapy in molecularly selected *DDR2*-driven tumors.

Clinical practice points

- *DDR2* mutations occurs in 4% of squamous cell lung cancer
- *DDR2*-mutated tumors are not mutually exclusive with others driver gene alterations
- *DDR2* gene mutations are not associated with specific clinical characteristics and show no prognosis impact
- Further studies deciphering *DDR2* biology in lung SCC are warranted for developing future dedicated targeted therapies.

Figure and Tables legends :

Table 1. Overview of the 11 *DDR2* somatic mutations observed in a panel of 271 lung SCCs. (ID: Identification Number ; Refseq: NCBI Reference Sequence Database; Ref: reference acid nucleic ; AA: aminoacid; VAFs: Variant Allele Frequencies)**Figure 1.** (A) Distribution of mutations in the domain structure of the *DDR2* protein. Novel mutations are listed in the upper part of the figure. IXJM: intra-cellular juxta-membrane domain. EXJM: extra-cellular juxta-membrane domain. TM: trans-membrane domain. Mutations also reported as SNP in literature are indicated with *. Mutations reported predictive to clinical response on *DDR2*-inhibiting drugs are noted with †. (B) Diagram of *DDR2* exon 7 alteration in relation to the 5' splice site.

Table 2. Proportion of driver gene alterations in 271 lung SCCs.

Table 3. Baseline characteristics of the investigation cohort.

Figure 2. Heatmap of the genomic landscape clustered by *DDR2* mutational status (investigation cohort). Each column represent a patient (n=136).

Figure 3. Kaplan-Meier analysis. (A) Global cohort survival of the *DDR2*-mutant (n = 11) and *DDR2*-WT groups (n=125). (B) Overall survival in the advanced *DDR2*-mutant (n = 7) and *DDR2*-WT (n = 48) lung SCC subgroups. Log-rank test results are shown on the graphs along with the number of patients at risk during follow-up.

List of Appendix

Appendix 1. Flow Chart of the study

Appendix 2. Annotation of the identified *DDR2* SNV not called as mutation in the validation cohort

Appendix 3. Logistic regression analysis (*DDR2* as the dependent variable). Univariate analysis.

Appendix 4: Sequences of the primers used for *DDR2* gene sequencing and mean coverage achieved for each primer.

Appendix 5: Sequencing coverage of *DDR2* gene

Author contributions

AL, HL, JM, BD and CR contributed to conception and design. CR, AL, HL and BD were responsible for the literature review. FL was responsible of pathology reviewing. CR and MK were responsible for figures. CR was responsible for clinical data collection. AL, MDT, AF, AA, HH and JM were responsible for molecular biology data collection, quality control and analysis. CR and MDT were responsible for statistical analysis. All authors were responsible for manuscript editing and final approval of the article. CR takes responsibility for the paper as a whole.

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Conflict of interest statement related to this work: None

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Table 1. Overview of the 11 DDR2 somatic mutations observed in a panel of 271 lung SCCs

ID	% tumoral cells	Exon	Ref	Alteration	Substitution Type	Chromosome	Starting codon	Nucleotide Change	AA Change	Type	VAFs (%)	VEST pathogenicity p-value	Functional impact
7915D	>50%	3	C	G	Transversion	1	162688915	c.62C>G	p.A21G	NS	43.21%	0,31110336	NEUTRAL
7135D	>50%	15	G	C	Transversion	1	162745442	c.1857G>C	p.R619S	NS	60.54%	0,01644484	DELETERIOUS
6313D	>50%	12	G	C	Transversion	1	162740194	c.1396G>C	p.G466R	NS	17.69%	0,15951798	NEUTRAL
5914D	25-50%	8	G	T	Transversion	1	162729635	c.721G>T	p.D241Y	NS	28.23%	0,00665495	DELETERIOUS
4789D	25-50%	16	G	A	Transition	1	162746132	c.2255G>A	p.R752H	NS	50,15%	0,00870242	DELETERIOUS
3605D	>50%	12	C	T	Transition	1	162740272	c.1474C>T	p.P492S	NS	39.36%	0,12656227	NEUTRAL
14390D	>50%	16	G	A	Transition	1	162742032	c.1723G>A	p.G575R	NS	15.79%	0,00743213	DELETERIOUS
13405D	25-50%	5	C	A	Transversion	1	162724630	c.402C>A	p.N134K	NS	12.32%	0,25879581	NEUTRAL
12428D	25-50%	7	G	C	Transversion	1	162725453	c.566-1G>C	NA	SV	30.59%	0,02006063	DELETERIOUS
12233D	>50%	5	G	T	Transversion	1	162724481	c.253G>T	p.E85X	SG	29.58%	0,00085979	DELETERIOUS
10113D	>50%	16	C	A	Transversion	1	162745961	c.2084C>A	p.A695D	NS	25.13%	0,00574364	DELETERIOUS

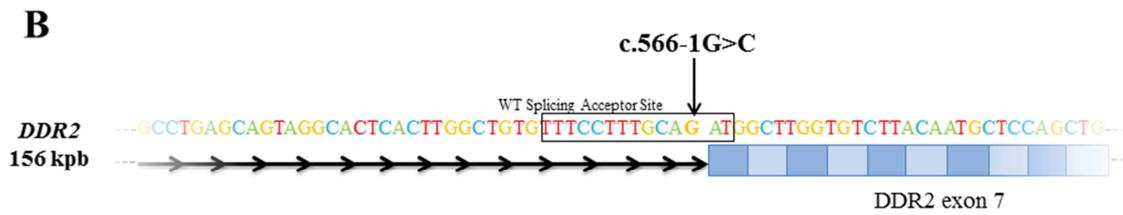
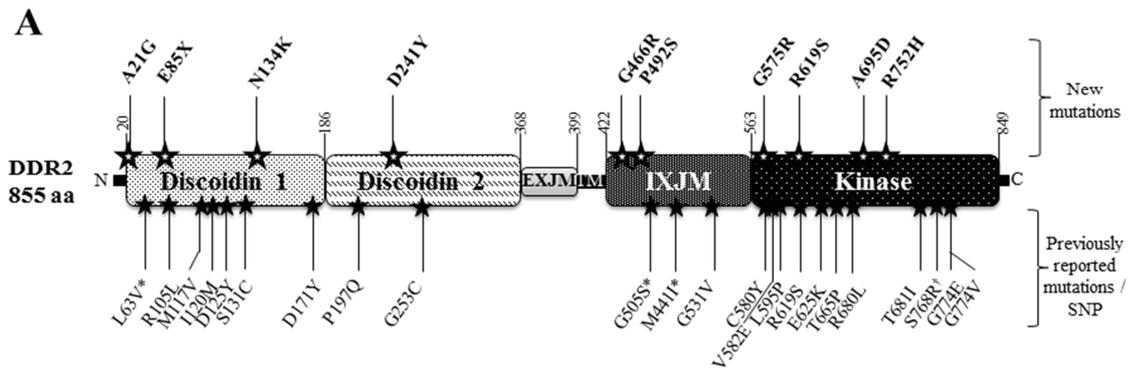
Table 2. Proportion of driver gene alterations in 271 lung SCCs

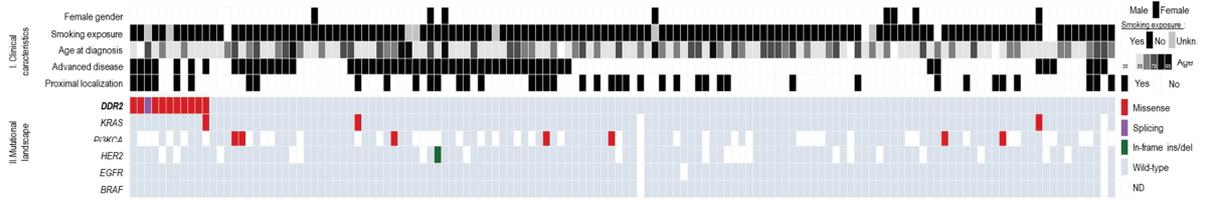
Gene	Analyzed samples	Mutated samples	%
DDR2 mutation	271	11	4,06
KRAS exon 2 mutation	265	5	1,89
PI3KCA exon 10 and 21 mutation	145	11	7,59
HER2 exon 20 insertion	234	1	0,43
EGFR activating mutation	264	0	0
BRAF exon 15 mutation	263	0	0
No alteration found	265	-	97,78

Table 3. Baseline characteristics of the

investigation cohort.

		DDR2 mutated	DDR2 wild- type	<i>p</i>
N		11	125	
Age	Median	65	68	0.1528
	Range (min;max)	(51-78)	(39-92)	
Gender	Male	11 (100%)	117 (94%)	0.999
	Female	0 (0%)	8 (6%)	
Smoking status	Active smoker	5 (45%)	54 (44%)	0.2793
	Former smoker	4 (36%)	62 (49%)	
	None	0 (0%)	5 (4%)	
	Unknown	2 (18%)	4 (3%)	
Pack-Years	Median	53.62	42.04	0.1282
	Range (min;max)	(30;80)	(2;100)	
Comorbidities	Cardio-vascular	8 (72%)	77 (61%)	0.7481
	COPD	5 (45%)	25 (20%)	0.1277
	Malignancies	4 (36%)	25 (20%)	0.2654
ECOG Performance status	0	3 (26.5%)	35 (28%)	0.701
	1	5 (45%)	64 (51%)	
	2	3 (26.5%)	21 (17%)	
	≥3	0 (0%)	5 (4%)	
Stage TNM (2009)	I-II	3 (27%)	47 (37%)	0.1191
	IIIA-resectableIIIB	1 (9%)	30 (24%)	
	unresectable IIIB-IV	7 (63%)	48 (38%)	
Tumor localization	Central	6 (54.5%)	36 (28%)	0.0939
	Distal	5 (45.5%)	89 (71%)	
Line of chemotherapy	Mean	1.4	1.4	0.8103
	Range (min;max)	(1;2)	(1;4)	
Follow-up	Median	13	13	0.918
	Range (min;max)	(1;42)	(1;54)	





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